SECURITY CLASSIFICATION OF THIS PAGE

# REPORT DOCUMENTATION PAGE

1. AD-A266 517

2.

2b

1b. RESTRICTIVE MARKINGS

3. DISTRIBUTION/AVAILABILITY OF REPORT
Approved for public release; distribution is unlimited

4. PERFORMING ORGANIZATION REPORT NUMBER(S)
NMRI 93-32

5. MONITORING ORGANIZATION REPORT NUMBER(S)

6a. NAME OF PERFORMING ORGANIZATION
Naval Medical Research Institute

6b. OFFICE SYMBOL (If applicable)

7a. NAME OF MONITORING ORGANIZATION
Naval Medical Command

6c. ADDRESS (City, State, and ZIP Code)
8901 Wisconsin Avenue
Bethesda, MD 20889-5055

7b. ADDRESS (City, State, and ZIP Code)
Department of the Navy
Washington, DC 20372-5120

8a. NAME OF FUNDING/SPONSORING ORGANIZATION Naval Medical Research & Development Command

8b. OFFICE SYMBOL (If applicable)

9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER

8c. ADDRESS (City, State, and ZIP Code)
8901 Wisconsin Avenue
Bethesda, MD 20889-5044

10. SOURCE OF FUNDING NUMBERS

| PROGRAM ELEMENT NO. | PROJECT NO. | TASK NO. | WORK UNIT ACCESSION NO. |
|---|---|---|---|
| 62233N | MM33C30 | 005-1051 | DN249507 |

11. TITLE (Include Security Classification)
The T cell antigen receptor: Biochemical aspects of signal transduction

12. PERSONAL AUTHOR(S) Samelson LE; Siegel JN; Phillips AF; Garcia-Morales P; Minami Y; Klausner RD; Fletcher MC; June CH

| 13a. TYPE OF REPORT | 13b. TIME COVERED | 14. DATE OF REPORT (Year, Month, Day) | 15. PAGE COUNT |
|---|---|---|---|
| journal article | FROM _____ TO _____ | 1993 | 14 |

16. SUPPLEMENTARY NOTATION
In: Molecular mechanisms of immunological self-recognition. Edited by Frederick W. Alt, Henry J. Vogel. San Diego: Academic Press 1993 pp.55-68

| 17. COSATI CODES | | |
|---|---|---|
| FIELD | GROUP | SUB-GROUP |
| | | |
| | | |

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
T cell receptor; calcium; tyrosine kinase; lymphokine production

19. ABSTRACT (Continue on reverse if necessary and identify by block number)



20. DISTRIBUTION/AVAILABILITY OF ABSTRACT
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS

21. ABSTRACT SECURITY CLASSIFICATION
Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL
Phyllis Blum, Librarian

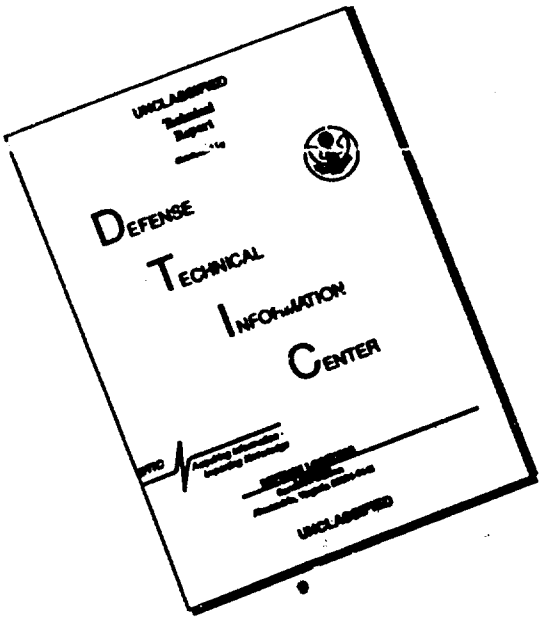
22b. TELEPHONE (Include Area Code)
(301) 295-2188

22c. OFFICE SYMBOL
MRL/NMRI

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

# DISCLAIMER NOTICE




THIS DOCUMENT IS BEST QUALITY AVAILABLE. THE COPY FURNISHED TO DTIC CONTAINED A SIGNIFICANT NUMBER OF PAGES WHICH DO NOT REPRODUCE LEGIBLY.

# 5

# THE T CELL ANTIGEN RECEPTOR: BIOCHEMICAL ASPECTS OF SIGNAL TRANSDUCTION

LAWRENCE E. SAMELSON[1], JEFFREY N. SIEGEL[1], ANDREW F. PHILLIPS[1], PILAR GARCIA-MORALES[1], YASUHIRO MINAMI[1], RICHARD D. KLAUSNER[1], MARY C. FLETCHER[2], AND CARL H. JUNE[2]

*1. Cell Biology and Metabolism Branch*
*National Institute of Child Health and Human Development*
*National Institutes of Health*
*Bethesda, Maryland 20892*
*and*
*2. Immune Cell Biology Program*
*Naval Medical Research Institute*
*Bethesda, Maryland 20814*

## INTRODUCTION

In the early 1980s the dogma defining T cell function was that activation of protein kinase C (PKC) and elevation of intracellular calcium were sufficient for cellular activation (1). As was observed in many other cellular systems, investigators were able to induce T cell lymphokine production and T cell proliferation by the addition of phorbol esters and calcium ionophores. These investigators thus concluded that the T cell antigen receptor was coupled to phospholipase C (PLC) and that activation of this enzyme would induce polyphosphoinositide (PI) hydrolysis with subsequent generation of diacylglycerol and inositol phosphates. These second messengers would then lead to activation of protein kinase C and elevation of intracellular calcium. Receptor



*Molecular Mechanisms of Immunological Self-Recognition*

regulation of these metabolites was viewed as critical to lymphokine gene transcription and cellular proliferation. Certainly, the activation of T lymphocytes by phorbol esters and calcium ionophores can be observed. However, there were some inconsistencies with this model. In the Jurkat system, investigators demonstrated that antigen receptor cross-linking by monoclonal antibody induced inositol phosphate production and calcium elevation. Presumably diacylglycerol was stoichiometrically produced after receptor cross-linking; nonetheless, investigators were forced to add phorbol esters to reconstitute full responses. Additional problems included the failure of the pharmacologic regimens to completely mimic the kinetics of T cell activation. There was always a concern that these pharmacologic agents might have multiple effects on the cells. The data that did most to question the adequacy of this model arose out of analysis of variants of the murine 2B4 hybridoma. Sussman *et al.* (2) were able to demonstrate a variant that produced interleukin-2 upon receptor cross-linking in the absence of demonstrable inositol phosphate release. Thus, although phosphoinositide breakdown is part of signaling in the T cell, its relationship to lymphokine production is not clear. Clearly, other pathways are activated through the T cell antigen receptor.

We took a different approach to questions of signal transduction in T cells. As of 1984 there had been many examples of ligand-induced receptor phosphorylation. For example, serine phosphorylation of the beta-adrenergic receptor induced by ligand had been demonstrated and related to receptor desensitization (3). Tyrosine phosphorylation of the epidermal growth factor (EGF) receptor and insulin receptors had been shown to be correlated with ligand binding and receptor kinase activation (4,5). In view of these studies pointing to the central role of protein kinase activitation in receptor-induced signal transduction, we asked whether the T cell antigen receptor was phosphorylated upon antigen engagement. To summarize several studies, we demonstrated that upon the addition of antigen, antireceptor antibody, or stimulatory anti–Thy-1 antibody, the antigen receptor is rapidly phosphorylated on two subunits (Fig. 1) (6–10). The CD3 γ chain is phosphorylated on serine residues, while the T cell receptor ζ chain is phosphorylated on tyrosine residues (11). The antigen-induced serine phosphorylation can be mimicked by addition of phorbol ester and is dependent on the presence of protein kinase C. The demonstration of tyrosine phosphorylation of a 21-kDa protein, later shown to be the T cell receptor ζ chain, was the first evidence for activation of a protein tyrosine kinase pathway in T lymphocytes. The receptor phosphorylations that we demonstrated could also be regulated by activation of a third kinase, the cyclic AMP–dependent protein kinase. Elevation of intracellular cyclic AMP levels resulted in inhibition of CD3 γ and T cell receptor ζ chain phosphorylation induced by antigen. This analysis of the phosphorylations occurring at the level of the receptor thus led to several insights. First, we demonstrated that

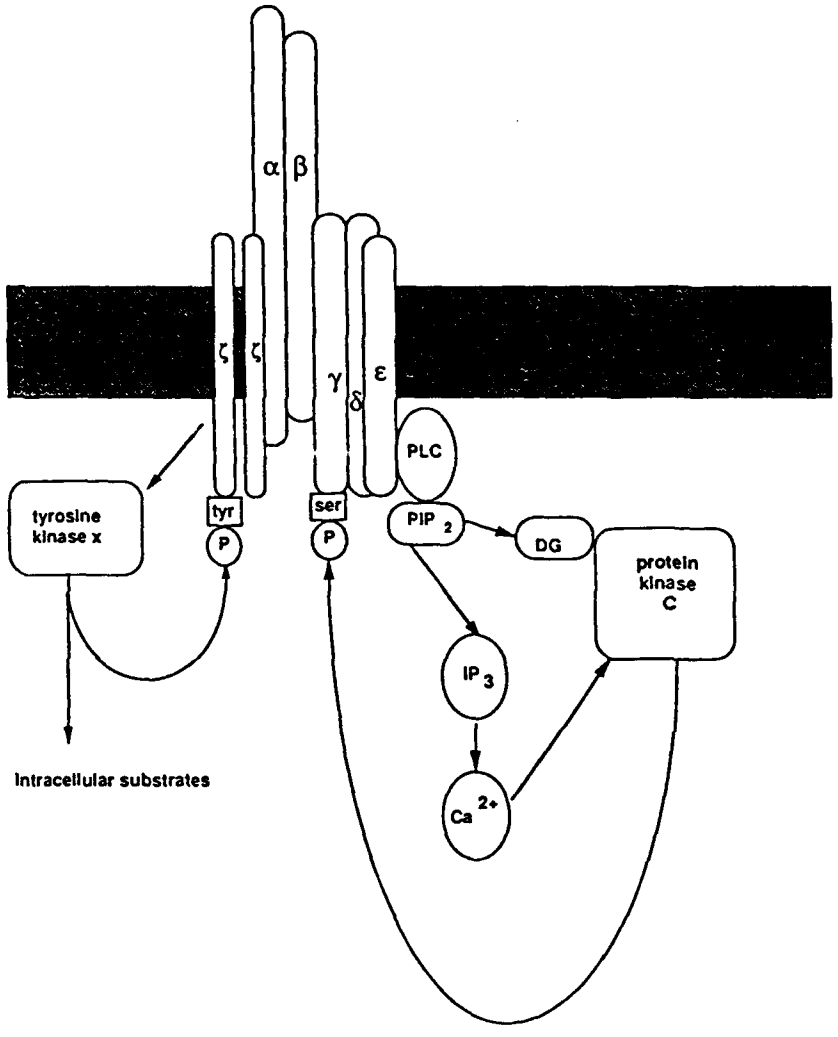



**Fig. 1.** A model defining the signal transduction pathways coupled to the T cell antigen receptor as described earlier (9).

multiple kinases were involved in regulation of receptor phosphorylation. Second, we confirmed that protein kinase C was activated through the antigen receptor, as had been predicted by other investigators. Third, we made the first demonstration of tyrosine kinase activation through the lymphocyte antigen receptor. It should be noted as well that since the antigen receptor subunits have

now all been cloned and sequenced, it is clear that the antigen receptor itself cannot encode the tyrosine kinase. The T cell receptor system was thus the first example of receptor-mediated activation of a nonreceptor tyrosine kinase that must be associated with or coupled to the receptor. Finally, we addressed the question of whether the two kinase pathways, serine and tyrosine, are activated in parallel or in series. We demonstrated that depletion of protein kinase C had no effect on activation of the tyrosine kinase. However, this left open the question of whether the kinases are activated in parallel or whether the tyrosine kinase pathway could activate the serine kinase. This question has been addressed more recently (see below).

## SUBSTRATE ANALYSIS IN MURINE AND HUMAN T CELLS; POSSIBLE TYROSINE KINASE REGULATION OF PHOSPHOLIPASE C

As described above, evidence for activation of a tyrosine kinase pathway in T cells was first provided by the observation that a 21-kDa protein associated with the antigen receptor is phosphorylated upon receptor occupancy. To further characterize the tyrosine kinase pathway in T cells, we have made extensive use of the technique of immunoblotting with specific antiphosphotyrosine antibodies. We have used this method to detect the phosphorylation of additional cellular substrates following receptor cross-linking (12). Comparison of lysates from unactivated and activated cells showed that phospho-ζ was detected in preparations from activated cells. In addition, we saw the phosphorylation of a 62-kDa protein and phosphorylation of proteins in the range 100–120 kDa. Further analysis by two-dimensional gel systems showed that the particular means of activation resulted in different patterns of substrate phosphorylation. Using the stimulatory anti-Thy-1 antibody, the ζ chain and 62-kDa substrates were the most prominent phosphoproteins. However, with antireceptor antibody or antigen, we detected relatively less 62-kDa phosphoproteins and more 53-kDa tyrosine phosphorylated proteins. The most impressive results of these studies were those of the kinetic analysis of tyrosine phosphorylation. Tyrosine phosphorylation of both the 62-kDa protein and the 53-kDa protein was very rapid with half-maximal phosphorylation by 2 min. Both half-maximal phosphorylation and the first detected phosphorylations preceded ζ chain tyrosine phosphorylation, which was not detected until 5 min and was half-maximal at about 15 min. This latter result suggested that the activation of tyrosine phosphorylation is very rapid and that, perhaps, phosphorylation of ζ chain represents a secondary and possibly regulatory event.

We have continued this characterization of the tyrosine kinase pathway by analyzing substrates in human T cells (13). We have used the Jurkat T cell

tumor line and, in addition, highly purified populations of normal peripheral blood T cells. The advantage of the latter source is that results reflect the status of the pathway in highly homogeneous, quiescent, $G_0$ lymphocytes. In both the tumor line and the resting cells, the addition of anti-CD3 monoclonal antibodies results in a specific increase in tyrosine phosphorylation of a number of cellular proteins. A careful kinetic analysis demonstrated that phosphorylation of one of these proteins, a 135-kDa protein, could be detected as early as 5 sec after receptor ligation. The tyrosine phosphorylation of a 100-kDa substrate could be first detected at 15 sec and was already maximal at 45 sec. As observed in the murine system, tyrosine phosphorylation of the human T cell receptor $\zeta$ chain was much slower. The very rapid kinetics of substrate phosphorylation were compared to the kinetics of both intracellular calcium elevation and production of inositol phosphates. Both of these had been measured in many laboratories previously and were thought to be among the most rapid events following T cell receptor ligation. Direct comparison of calcium, PI turnover, and tyrosine phosphorylation of the substrates, however, revealed that phosphorylation of the 135- and 100-kDa proteins preceded detectable elevations in calcium or inositol phosphates. These kinetic relationships suggested that tyrosine phosphorylation might regulate inositol phosphate release. In this regard, the results were reminiscent of results discussed above for the EGF receptor and platelet-derived growth factor (PDGF) receptor systems, in which tyrosine phosphorylation of phospholipase C $\gamma$ is thought to regulate enzymatic function (Fig. 2).

To address this question in T cells, we performed a series of studies with the tyrosine kinase inhibitor herbimycin (14). This drug was initially isolated as a reagent that could induce reversion of v-*src*–transformed cells. Addition of the reagent to T cells resulted in inhibition of the ability to induce tyrosine phosphorylation as detected by a lack of substrate phosphorylation. Surprisingly, the drug required at least 8 hours for maximal activity. The explanation for this time requirement was shown to be related to the effect of the drug on the level of tyrosine kinase protein. Treatment with herbimycin results in loss of lck protein, a process that might require this long incubation. The absence of kinase protein can obviously be detected as a loss of activity. Fyn and lck tyrosine kinase activities were reduced with the same kinetics and dose dependence as the inhibition of substrate tyrosine phosphorylation. The specificity of these effects was demonstrated by analyzing the level of c-*raf* serine kinase activity and protein. As will be described below, this kinase is activated in T cells by stimulation of protein kinase C. We demonstrated that there was a minimal effect on the level of PKC-induced *raf* activity and no loss of *raf* protein in response to overnight herbimycin treatment. This result demonstrates that there was minimal effect of the drug on the two serine kinases, PKC and *raf*. The functional effect of herbimycin treatment was then analyzed. Herbimycin was shown to inhibit

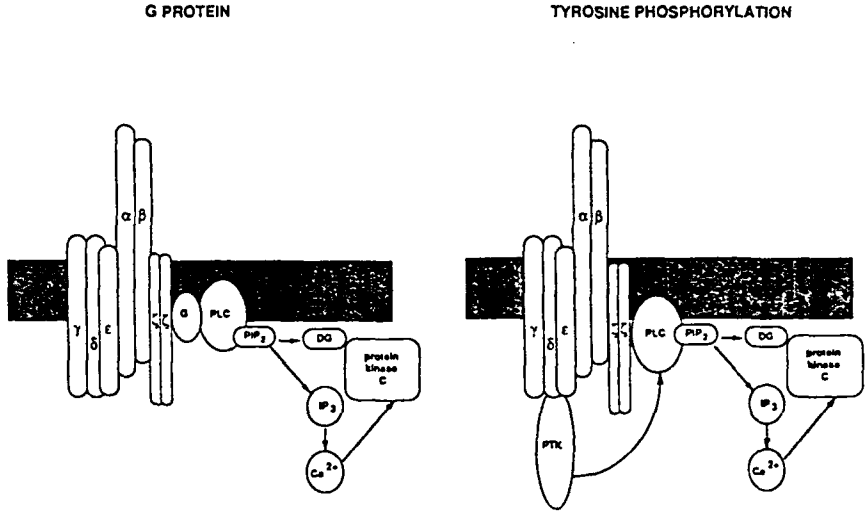



**Fig. 2.** Alternative models for describing T cell antigen–receptor coupling to phospholipase C. The first activation of the receptor activates a guanine nucleotide-binding protein (G protein) that then interacts with phospholipase C, inducing its activation. In the second model we propose that activation of a tyrosine kinase results in tyrosine phosphorylation of phospholipase C or associated proteins, resulting in activation of the enzyme and polyphosphoinositide hydrolysis.

both inositol phosphate production and receptor-mediated calcium elevation. Addition of aluminum fluoride to these cells resulted in the expected induction of calcium elevation. Since this reagent presumably activates phospholipase C via a G protein, this result indicates that this enzyme is intact unless herbimycin directly inhibits the T cell receptor (TCR)–coupled PLC. We believe it more likely instead that tyrosine phosphorylation is required for activation of PLC via TCR ligation. As a further control, we analyzed the effect of herbimycin on such late T cell activation events as interleukin-2 (IL-2) production and IL-2 receptor expression. The former can be induced by the combination of phorbol myristate acetate (PMA) and ionomycin, while the latter, IL-2 receptor up-regulation, occurs with PMA alone. Neither of these transcriptionally regulated events was significantly affected by herbimycin. However, the drug entirely prevented CD3-induced IL-2 production and CD3-induced IL-2 receptor up-regulation. It is more difficult to ascribe inhibition of these late events to decreased tyrosine phosphorylation of substrates, but it certainly is a likely possibility. Thus, use of this drug further supports the kinetic arguments presented above; that is, tyrosine phosphorylation is important for phospholipase C

activation or for phospholipase C regulation. Similar conclusions have been reached by Mustelin *et al.* (15) using the tyrosine kinase inhibitor genistein.

## ANALYSIS OF TYROSINE PHOSPHATASES

Regulation of tyrosine phosphorylation by the action of tyrosine phosphatases has become of interest recently due to the recognition that CD45 is a tyrosine phosphatase (16). We have taken two approaches to the analysis of CD45 activity in T cells. In human T cells, as noted above, T cell stimulation induced via either the CD2 or CD3 molecules can be abrogated by coaggregation with the CD45 molecule using chemically cross-linked, heteroconjugate monoclonal antibodies (17). We have demonstrated that CD2 induces an increase in tyrosine phosphorylation in T cells and have confirmed that CD2–CD45 heteroconjugate antibodies inhibit calcium flux in these cells (18). We predicted that polyphosphoinositide hydrolysis would also be inhibited using these antibody heteroconjugates, and indeed we found that production of inositol phosphates $IP_1$, $IP_2$, $IP_3$ and $IP_4$ was markedly decreased. We then asked what the effect was on substrate tyrosine phosphorylation under these circumstances. CD2 stimulation results in tyrosine phosphorylation of 135-kDa and 100-kDa phosphoproteins, as was demonstrated with anti-CD3 stimulation. When one cross-links CD45 molecules via antibody-induced cross-linking, one sees an increase in tyrosine phosphorylation only of the 135-kDa protein. The same result is observed when one forms heteroconjugates between CD2 and CD45 with biotinylated antibodies and avidin. We detect an increase in phosphorylation of the 135-kDa protein without phosphorylation of the 100-kDa protein. We can perform the experiment in another fashion with the same result. In this case, one uses a pair of stimulatory anti-CD2 antibodies for activation and detects tyrosine phosphorylation of both substrates. When one adds to this combination anti-CD45 antibodies, which are biotinylated with avidin, one decreases the intensity and delays the onset of tyrosine phosphorylation of the 100-kDa protein. These results have several implications. First, we have provided further evidence that manipulation of the tyrosine phosphorylation pathway, this time by perturbation of a tyrosine phosphatase, affects phospholipase C activity. Second, we have demonstrated that tyrosine phosphorylation of a 100-kDa protein is correlated with PLC activity. Perhaps CD45 in some way regulates activity of the enzyme, for example, by controlling its level of tyrosine phosphorylation. Finally, the 100-kDa protein, perhaps, is a direct substrate for CD45.

A second approach to analyzing the role of tyrosine phosphatases was performed using the drug phenylarsine oxide (PAO). Investigators studying the

insulin receptor system have shown that this reagent inhibits tyrosine phosphatases (19,20). When adipocytes or fibroblasts are preincubated with this drug prior to insulin treatment, one detects tyrosine-phosphorylated substrates that are otherwise not seen. The substrates presumably are tyrosine phosphorylated during ligand occupancy. However, normally, in the absence of phenylarsine oxide, tyrosine phosphate is turned over very rapidly. We asked what would happen when this drug was added to the murine hybridoma 2B4 (21). Initially, we demonstrated that PAO can inhibit tyrosine phosphatase activity of the CD45 molecule. CD45 was isolated with monoclonal antibodies, and the effect of phenylarsine oxide on its *in vitro* tyrosine phosphatase activity was tested. We demonstrated that PAO at concentrations between 5 and 10 μM resulted in 50% inhibition of phosphatase activity. We then demonstrated that the drug had no effect on the fyn or lck tyrosine kinases. Although there are certainly other tyrosine phosphatases and kinases in the cell, we use these data to support the assumption that the drug is working preferentially, if not solely, against tyrosine phosphatases and not kinases. The effects of the drug on the tyrosine phosphorylation of substrates are complex. Addition of PAO to unstimulated T cells results in a dose-dependent increase in tyrosine phosphorylation on a number of substrates that are otherwise not detected. This indicates either that there is a constitutively active tyrosine kinase in T cells or that PAO activates a tyrosine kinase. Of greater interest is the effect of the drug on stimulated T cells. At low concentrations, the drug appears to synergize with stimulation induced by the G7 anti-Thy-1 antibody. There is a dramatic increase in the induction of tyrosine phosphorylation of the T cell receptor ζ chain and of several other tyrosine kinase substrates of 54 and 62 kDa. The increase in phosphorylation of the 54-kDa substrate is nearly 14-fold. As the dose of PAO is increased, one observes inhibition of all activation-induced tyrosine phosphorylation. Thus, T cell receptor ζ chain tyrosine phosphorylation is completely inhibited, and the level of tyrosine phosphate on the 54- and 62-kDa substrates returns to the level induced by drug alone. These results have several implications. First, it appears that there is a constitutive level of tyrosine phosphatase activity in the cells which, when inhibited by low concentrations of PAO, results in detection of additional substrates and enhanced levels of tyrosine phosphorylation induced through the Thy-1 molecule. Second, the results of the experiments with the high concentrations of PAO indicate that tyrosine phosphatases may be involved in the regulation of tyrosine kinases. Inhibition of the phosphatase results in failure of tyrosine kinase activation, as assessed by substrate phosphorylation. In this way, the phenylarsine oxide experiments are compatible with the experiments of Pingel and Thomas, described above, in which the CD45 molecule is genetically absent (22). When CD45-negative cells are reconstituted with CD45 cDNA, TCR-mediated signaling is restored (23).

## THE SERINE KINASE PATHWAY

The serine/threonine kinase c-*raf* has been identified as a substrate for the PDGF and EGF receptor tyrosine kinases. As described above, in a number of recent studies c-*raf* has been demonstrated to bind to activated PDGF or EGF receptor kinases upon ligand activation of those receptors. Moreover, c-*raf* has been shown to be phosphorylated on serine and, in some cases, tyrosine residues upon ligand addition. These phosphorylations induce activation of the c-*raf* kinase. We began to investigate c-*raf* as a possible substrate in the T cell system. We reasoned that since the T cell antigen receptor was coupled to both serine and tyrosine kinase pathways, c-*raf* could serve as a potential integrator of both kinases. We thus predicted that we could detect both serine and tyrosine phosphorylation of *raf*. We used specific anti-*raf* antibodies to immunoprecipitate *raf* from $^{32}$P-labeled T cells that were either left unstimulated or stimulated with antireceptor antibodies, anti–Thy-1 antibodies, or phorbol esters (24). Under all these stimulatory conditions, we detected an increase in phosphate labeling of immunoprecipitated c-*raf* and a shift in c-*raf* migration on an SDS-PAGE gel so that it migrated with a greater apparent molecular weight.

To determine which kinase pathway activated c-*raf* phosphorylation, we performed phosphoamino acid analysis of isolated c-*raf*. We detected only serine and, to a lesser degree, threonine phosphorylation of c-*raf*. To confirm the absence of phosphotyrosine, we obtained the antiphosphotyrosine monoclonals used by Morrison *et al.* (25) to detect tyrosine phosphorylation of c-*raf* following PDGF treatment. These reagents were able to detect an increase in tyrosine phosphorylation on a number of substrates following T cell activation. One substrate was a 70-kDa protein which was a candidate for c-*raf*. However, anti-*raf* antibodies failed to immunoprecipitate any tyrosine-phosphorylated substrate. It thus seemed unlikely that the tyrosine kinase activated by the T cell antigen receptor was responsible for the hyperphosphorylation of *raf*. We then asked whether protein kinase C activation could be responsible. We depleted the cells of PKC with a high dose of phorbol ester and demonstrated that in cells treated in this fashion there was no further phorbol ester– or anti–Thy-1–stimulated *raf* phosphorylation. High-resolution phosphopeptide mapping was used to compare the pattern of phosphorylation induced by phorbol ester, anti–Thy-1, and antireceptor antibodies. A number of phosphopeptides were detected in the unactivated state, and several new ones were detected with stimulation. In all stimulatory circumstances, the pattern of phosphorylation was similar. In addition, in variants of 2B4 in which there was a failure of PI hydrolysis upon stimulation, there was a concomitant lack of *raf* activation.

The combination of phosphoamino acid analysis, phosphopeptide mapping, the analysis of 2B4 variants, and the PKC depletion experiments led us to the

conclusion that the mechanism for phosphorylation of c-*raf* in T cells is due to activation of protein kinase C. The consequences of c-*raf* phosphorylation were then tested in *in vitro* kinase assays. Immunoprecipitates containing *raf* from unactivated and activated cells were incubated with histone H1 or H5 as exogenous substrates. c-*raf* isolated from activated cells induced an increase in phosphate incorporation into histone, as well as into a 120-kDa substrate which appears to coimmunoprecipitate with *raf*. Thus, stimulation of *raf* phosphorylation correlates with the increase in activity of the enzyme. In fact, dephosphorylation of the activated *raf* molecule by alkaline phosphatase resulted in inactivation of its kinase activity. These studies allow us to propose a model in which antigen receptor occupancy results in a cascade of events. Activation of phospholipase C presumably via the tyrosine phosphorylation pathway, as described above, leads to polyphosphoinositide hydrolysis, activation of protein kinase C with subsequent phosphorylation, and activation of the c-*raf* protein serine/threonine kinase. The function of activated *raf* remains to be determined. However, it is of note that activated c-*raf* appears to translocate to the nucleus, and activated forms of *raf* have been demonstrated to increase transcriptional activity of several genes.

## FYN IS A T CELL RECEPTOR–ASSOCIATED TYROSINE KINASE

We have attempted to identify the kinase responsible for tyrosine phosphorylation of the T cell receptor ζ chain and phosphorylation of the other cellular substrates for several years. One candidate is lck, a member of the *src* kinase family. This tyrosine kinase is a T cell–specific kinase, which has recently been shown to be associated with the CD4 and CD8 molecules (26,27). However, for a number of reasons it is unlikely that lck is solely responsible for T cell receptor–induced tyrosine phosphorylation. For one, a number of cells are CD4 and CD8 negative. These include the γ-δ cells detected in thymus and periphery. In addition, the 2B4 hybridoma that we use extensively in our studies is currently CD4 negative. It is, of course, possible that lck is associated with yet another cell surface molecule such as the antigen receptor. Most problematic, however, is the observation that CD3 cross-linking, which results in T cell receptor activation and ζ chain phosphorylation, fails to result in lck activation (28). In contrast, cross-linking of CD4 or CD8 molecules results in lck activation, as demonstrated by increased autophosphorylation and phosphorylation of an exogenous substrate. Finally, the pattern of tyrosine-phosphorylated substrates differs whether one activates through the T cell antigen receptor or through the CD4 or CD8 molecule (29). For these reasons, we thought that yet another tyrosine kinase could be responsible for the tyrosine phosphorylations that we detect.

Several years ago we had attempted to demonstrate a kinase associated with the antigen receptor using immune complex kinase assays. These experiments were unsuccessful. However, about 1 year ago we repeated these experiments using digitonin instead of Triton X-100 as our means of solubilizing the antigen receptor. Immunoprecipitates using antireceptor antibodies were then incubated with kinase buffer containing radioactive [$\gamma$-$^{32}$P]ATP and the necessary cations for assaying protein tyrosine kinases. This protocol resulted in an impressive level of phosphorylation of several proteins specifically coimmunoprecipitated with antireceptor antibodies (30). The phosphoproteins have apparent molecular masses of 130, 120, 59, 56, 28, and 21 kDa. We failed to see a similar pattern of phosphoproteins when we used anti-H2, anti-LFA, or anti-CD45 antibodies. This pattern of phosphorylation was observed with anti–T cell receptor $\alpha$ antibodies (A2B4-2) or three anti-$\varepsilon$ antibodies. Antibodies recognizing the carboxyl-terminal determinants of CD3-$\delta$ or T cell receptor $\zeta$ failed to immunoprecipitate proteins which were phosphorylated in a similar fashion. This result suggested that the kinase activity we were precipitating was associated with cytoplasmic domains of the antigen–receptor complex. We interpreted these results to indicate that a protein kinase was coprecipitated with the antigen receptor. We then determined that these phosphoproteins were predominantly phosphorylated on tyrosine residues. The lower-molecular-mass proteins (at 21 and 28 kDa) were exclusively tyrosine phosphorylated. The phosphoproteins at 56, 59, 120, and 130 kDa were phosphorylated on tyrosine and, to a lesser extent, serine residues. The identity of the lower-molecular-mass proteins was determined by two-dimensional diagonal gel analysis. The prominent phosphoproteins that we detected were phosphorylated T cell receptor $\zeta$ chains. The CD3 $\gamma$, $\delta$, and $\varepsilon$ chains were also tyrosine phosphorylated, but to a lesser extent.

To determine which protein tyrosine kinase was responsible for these tyrosine phosphorylation events, we compared the pattern of phosphoproteins that we detected with anti-CD3 $\varepsilon$ antibodies and with a panel of antibodies binding to tyrosine kinases (27,31). By direct immunoprecipitation we determined that there were high levels of fyn and yes activity in these T cells and a low amount of lck activity. The pattern of phosphorylation that we detected with the anti-fyn antibodies was virtually identical to that which we saw with anti–CD3 $\varepsilon$ precipitation. In particular, we observed phosphorylation of the 56-, 59-, 120-, and 130-kDa proteins, as well as low-molecular-mass proteins. When we looked at the phosphoproteins detected after anti-fyn immunoprecipitation on a two-dimensional diagonal gel, we again noted that we detected T cell receptor $\zeta$ chain phosphorylation as well as CD3 phosphorylation. Therefore, the pattern of phosphoproteins we detected with anti-TCR monoclonal antibodies was most like that seen with anti-fyn antibodies; moreover, anti-fyn antibodies immunoprecipitated the antigen receptor (Fig. 3). It should be noted that the fyn

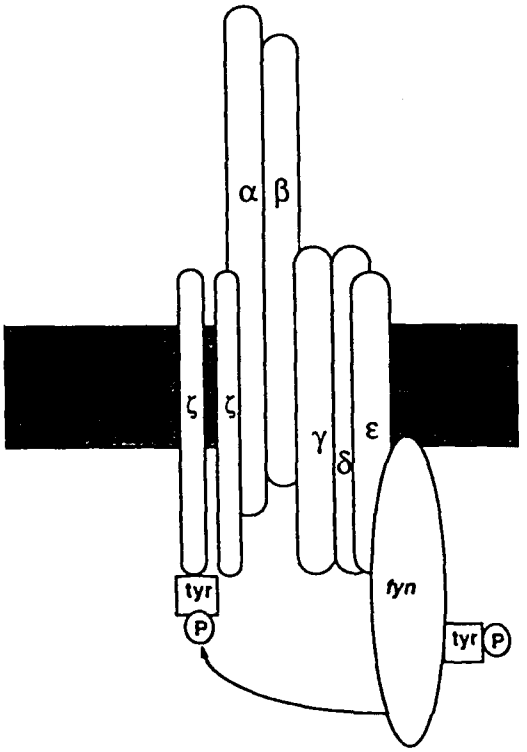



**Fig. 3.** The T cell antigen receptor is coupled to the fyn kinase. In *in vitro* kinase assays fyn is autophosphorylated and phosphorylates the antigen receptor ζ chain on tyrosine residues. There is no evidence to date that fyn is specifically associated with the CD3 ε chain. The depiction of the fyn–CD3 ε association in this model is purely arbitrary.

present in T cells differs from that in fibroblasts, suggesting that T cell fyn has a specific function (32).

Further confirmation that fyn is the kinase associated with the receptor came from two additional experiments. In one, we performed the *in vitro* kinase reaction after antireceptor antibody immunoprecipitation. This immune complex was then eluted with the detergent Nonidet P-40 (NP-40), which is known to disrupt the antigen receptor components. This detergent eluate was then subjected to reimmunoprecipitation with anti-lck and anti-fyn antibodies. We detected a 59-kDa phosphoprotein only with anti-fyn immunoprecipitation. Second, we performed a number of phosphopeptide analyses comparing the patterns of the phosphoproteins immunoprecipitated with antireceptor and

anti-fyn antibodies. Digestion of the 130-, 120-, and 56-kDa phosphoproteins with staphylococcal V8 protease resulted in an identical pattern of phosphopeptides, whether one immunoprecipitated with anti-fyn or anti-receptor antibodies. The 59-kDa protein, which we believe is the fyn kinase itself, was also subjected to protease digestion. Fyns isolated directly from fibroblasts, from T cells, and via the antigen receptor from T cells share a number of peptides. There are subtle differences between the patterns of fyn isolated directly from the T cell and via the antigen receptor. We have performed further studies with trypsin digestion and high-resolution two-dimensional analysis. In these studies we demonstrate that both directly precipitated fyn and indirectly precipitated fyn share a common tyrosine-phosphorylated peptide, the site of autophosphorylation. Fyn isolated from T cells via the antigen receptor contains several additional phosphopeptides which are phosphorylated on both tyrosine and serine residues. We are currently analyzing the stoichiometry of interaction and the question of whether fyn is activated via the antigen receptor.

## REFERENCES


1. Weiss, A., Imboden, J., Hardy, K., Manger, B., Terhorst, C., and Stobo, J. (1986) *Annu. Rev. Immunol.* **4**, 593–619.
2. Sussman, J. J., Mercep, M., Saito, T., Germain, R. N., Bonvini, E., and Ashwell, J. D. (1988) *Nature* **334**, 625–628.
3. Sibley, D. R., Benovic, J. L., Caron, M. C., and Lefkowitz, R. J. (1987) *Cell* **48**, 913–922.
4. Cohen, S., Carpenter, G., and King, L., Jr. (1980) *J. Biol. Chem.* **255**, 4834–4842.
5. Kasuga, M., Karlsson, F. A., and Kahn, C. R. (1982) *Science* **215**, 185–187.
6. Samuelson, L. E., Harford, J. B., Schwartz, R. H., and Klausner, R. D. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 1969–1973.
7. Samelson, L. E., Patel, M. D., Weissman, A. M., Harford, J. B., and Klausner, R. D. (1986) *Cell* **46**, 1083–1090.
8. Samelson, L. E., O'Shea, J. J., Luong, H. T., Ross, P., Urdahl, K. B., Klausner, R. D., and Bluestone, J. (1987) *J. Immunol.* **139**, 2708–2714.
9. Patel, M. D., Samelson, L. E., and Klausner, R. D. (1987) *J. Biol. Chem.* **262**, 5831–5838.
10. Klausner, R. D., O'Shea, J. J., Luong, H., Ross, P., Bluestone, J. A., and Samelson, L. E. (1987) *J. Biol. Chem.* **262**, 12654–12659.
11. Baniyash, M., Garcia-Morales, P., Luong, E., Samelson, L. E., and Klausner, R. D. (1988) *J. Biol. Chem.* **263**, 18225–18230.
12. Hsi, E. D., Siegel, J. N., Minami, Y., Luong, E. T., Klausner, R. D., and Samelson, L. E. (1989) *J. Biol. Chem.* **264**, 10836–10842.
13. June, C. H., Fletcher, M. C., Ledbetter, J. A., and Samelson, L. E. (1990) *J. Immunol.* **144**, 1591–1599.
14. June, C. H., Fletcher, M. C., Ledbetter, J. A., Schieven, G. L., Siegel, J. N., Phillips, A. F., and Samelson, L. E. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 7722–7726.
15. Mustelin, T., Coggeshall, K. M., Isakov, N., and Altman, A. (1990) *Science* **247**, 1584–1587.
16. Charbonneau, H., Tonks, N. K., Walsh, K. A., and Fischer, E. H. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7182–7186.

17. Ledbetter, J. A., Tonks, N. K., Fischer, E. H., and Clark, E. A. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8628–8632.
18. Samelson, L. E., Fletcher, M. C., Ledbetter, J. A., and June, C. H. (1990) *J. Immunol.*, in press.
19. Bernier, N., Laird, D. M., and Lane, M. D. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1844–1848.
20. Levenson, R. M., and Blackshear, P. J. (1989) *J. Biol. Chem.* **264**, 19954–19993.
21. Garcia-Morales, P., Luong, E. T., Minami, Y., Klausner, R. D., and Samelson, L. E. (1990) *Proc. Natl. Acad. Sci. USA*, in press.
22. Pingel, J. T., and Thomas, M. L. (1989) *Cell* **58**, 1055–1065.
23. Koretzky, G. A., Picus, J., Thomas, M. L., and Weiss, A. (1990) *Nature* **346**, 66–68.
24. Siegel, J. N., Klausner, R. D., Rapp, U., and Samelson, L. E. (1990) *J. Biol. Chem.* **265**, 18472–18480.
25. Morrison, D. K., Kaplan, D. R., Escobedo, J. A., Rapp, U. R., Roberts, T. M., and Williams, L. T. (1989) *Cell* **58**, 649–657.
26. Rudd, C. E., Trevillyan, J. M., Dasgupta, J. D., Wong, L. L., and Schlossman, S. F. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5190–5194.
27. Veillette, A., Bookman, M. A., Horak, E. M., and Bolen, J. B. (1988) *Cell* **55**, 301–308.
28. Veillette, A., Bookman, M. A., Horak, E. M., Samelson, L. E., and Bolen, J. B. (1989) *Nature* **338**, 257–259.
29. Veillette, A., Bolen, J. B., and Bookman, M. B. (1989) *Mol. Cell. Biol.* **9**, 4441–4446.
30. Samelson, L. E., Phillips, A. F., Luong, E. T., and Klausner, R. D. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 4358–4362.
31. Horak, I. D., Kawakami, T., Gregory, F., Robbins, K. C., and Bolen, J. B. (1989) *J. Virol.* **63**, 2343–2347.
32. Cooke, M. P., and Perimutter, R. M. (1990) *New Biol.* **1**, 66–74.